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IMUNOHISTOHEMIJSKE I KARIOMETRIJSKE RAZLIKE I SLIČNOSTI TUMORA PLJUVAČNIH ŽLEZDI IZMEĐU PLEOMORFNOG ADENOMA, ADENOMA BAZALNIH ĆELIJA I POLIMORFNOG ADENOKARCINOMA NISKOG GRADUSA

IMMUNOHISTOCHEMICAL AND KARYOMETRIC SIMILARITIES AND DIFFERENCES OF SALIVARY GLAND TUMORS BETWEEN PLEOMORPHIC ADENOMA, BASAL CELL ADENOMA AND POLYMORPHOUS LOW GRADE ADENOCARCINOMA

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Sažetak

Uvod: *Tumori pljuvačnih žlezda su veoma retke neoplazme. S obzirom na njihovu patohistološku sliku, ovi tumori predstavljaju veoma veliki dijagnostički izazov.*
Cilj: *istaživanja je diferencijacija ova tri tipa tumora primenom imunohistohemijske i morfometrijske analize, kao i određivanje visine Ki67 proliferativnog indeksa.*
Materijal i metode: *Istraživanje je obuhvatilo 44 tumora, 20 pleomorfnih adenoma, 12 adenoma bazalnih ćelija i 12 polimorfnih adenokarcinoma niskog gradusa. Analizirana je ekspresija Ki67, p53 i HER-2 antigena, kao markera proliferacije. U sklopu diferencijalne dijagnostike, analizirana je ekspresija CEA, EMA, GFAP, p63, vimentina, CK14, α-SMA, S-100 protein i WT1 antigena. Morfometrijska analiza vršena je u softverskom paketu „ImageJ" verzija 1.43u.*
Rezultati: *Neoplastične ćelije u pleomorfnom adenomu su pokazale jaku ekspresiju GFAP, p63, WT1, vimentin i S100. U grupi od dvanaest polimorfnih adenokarcinoma niskog gradusa prisutna je difuzna ekspresija CK14, S100, vimentin i EMA su bili apsolutno eksprimirani, dok je αSMA bio negativan. Adenom bazalnih ćelija pokazuje pozitivnost na S-100, CEA, p63 i vimentin. Analizom vrednosti proliferativnog Ki67 indeksa ustanovljena je statistički značajna razlika u grupi pleomorfnog adenoma, što se dovodi u vezu sa čestim recidiviranjem. Morfometrijskom analizom se uočavaju veće vrednosti u grupi polimorfnog adenokarcinoma niskog gradusa, ali su statistički značajne razlike nađene samo za Feretov dijametar i integrisanu optičku gustinu u odnosu na pleomorfni adenom ($p<0,05$). U grupi adenoma bazalnih ćelija tumorske ćelije su pokazale statistički veće vrednosti za integrisanu optičku gustinu u odnosu na pleomorfni adenom ($p<0,001$).*
Zaključak: *Za diferencijalnu dijagnozu tumora pljuvačnih žlezda, pored osnovne mikromorfološke, neophodna je i imunohistohemijska i morfometrijska analiza.*

Ključne riječi: pleomorfni adenom, polimorfni adenokarcinom niskog gradusa, adenom bazalnih ćelija, imunohistohemija, morfometrija

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Abstract

Introduction: *Salivary gland tumors are extremely rare neoplasms. Given their pathohistological image, these tumors represent a great diagnostic challenge.*
The aim *of our study was to differentiate these three tumor types by applying the immunohistochemical and morphometric analysis.*
Material and Methods: *The entire study was conducted at the Center for Pathology and Pathological Anatomy in Niš. The study included 44 tumors, 20 pleomorphic adenomas, 12 basal cells adenomas and 12 polymorphous low-grade adenocarcinomas. The expression of Ki67, p53 and HER-2 antigens, as proliferation markers, was analyzed. The differential diagnostics also included the analysis of the expression of CEA, EMA, GFAP, p63, vimentin, CK14, α-SMA, S-100 protein and WT1 antigen. The morphometric analysis was done in the program pack "ImageJ" version 1.43u. The statistical analysis of data was done in the program pack SPSS 15.0.*
Results: *Neoplastic cells in pleomorphic adenoma showed a strong expression of GFAP (20/20), p63 (20/20), WT1 (20/20), vimentin (18/20) and S100 (16/20). Diffuse expression of CK14 (12/12) was present in the group of 12 polymorphous low-grade adenocarcinomas. S-100, vimentin and EMA were absolutely expressed, whereas α-SMA was negative. Basal cell adenoma showed negativity to S-100, CEA, p63 and vimentin. The analysis of the proliferative Ki67 index values pointed to a statistically significant difference in the pleomorphic adenoma group, which was associated with a frequent recurrence of this benign tumor. The analysis of morphometric characteristics showed higher values in the polymorphous low-grade adenocarcinoma group, but statistically significant differences were found only for the Feret diameter and the integrated optical density ($p < 0.05$). As for the basal cell adenoma group, tumor cells showed statistically higher values for the integrated optical density ($p < 0.001$).*
Conclusion: *Apart from the basic micromorphological analysis, the differential analysis of salivary gland tumors also requires the immunohistochemical analysis as well as the monitoring of morphometric characteristics of these tumors' nuclei.*

Key words: pleomorphic adenoma, polymorphous low-grade adenoma, basal cell adenoma, immunohistochemistry, morphometry

Uvod

Tumori pljuvačnih žlezda su veoma retke neoplazme. S obzirom na njihovu patohistološku sliku, ovi tumori predstavljaju veoma veliki dijagnostički izazov. Učestalost ovih tumora se kreće od 3 do 6% svih tumora regije glave i vrata [1,2]. Parotidna žlezda je najčešće mesto tumora pljuvačnih žlezda, sa učestalošću od 80-85%, a skoro 75% ovih tumora su benigne neoplazme. Druga po učestalosti je submandibularna žlezda, sa 10%, ali polovina tumora ove lokalizacije su maligne neoplazme. Sa frekvencijom od 1% je sublingvalna žlezda, ali oko 80% tumora je maligno. Male pljuvačne žlezde su mesta sa najčešćom frekvencijom malignih tumora [3,4]. Epidemiološki podaci prikazuju različitu učestalost u različitim etničkim grupama i delovima sveta, što dodatno otežava globalnu incidenciju ovih tumora [5,6]. Prosečna starost bolesnika je 46-47 godina, ali pik pojedinih tumora je u šestoj i sedmoj deceniji života [2].

Pleomorfni adenom, označen još i kao mešoviti tumor (tumor mixtus), predstavlja najčešću neoplazmu pljuvačnih žlezda. Sreće se u svim životnim dobima, ali pik incidencije je u petoj deceniji. Jednako je zastupljen među polovima, sa blagom dominacijom kod žena. U 80% slučajeva lokalizovan je u parotidnoj žlezdi, i to u donjem polu. Ako je lokalizovan u dubokom režnju, ima prezentaciju parafaringealnog tumora; obično su sporog rasta i bezbolni su. Mali tumori se prezentuju kao glatke, čvrste, mobilne globule, dok tumori većih dimenzija mogu oštetiti kožu i sluzokožu koja ih prepokriva. Multifokalni i rekurentni tumori mogu biti fiksirani za okolno tkivo. Sporadično se mogu javiti sa drugim tumorima, naročito sa Warthin tumorom. Veličina tumora varira od 2 do 5 cm, ali mogu dostići i enormne dijametre. U slučajevima kada se javi infarceracija tumora, mogu se javiti bol i parestezije. Na nepcu se najčešće javljaju na prelazu između mekog i tvrdog nepca, kada mogu biti fiksirani, zbog blizine periosta [7,8].

Makroskopski su jasno definisani, okruglog do ovalnog oblika. Debljina kapsule varira, a može i nedostajati, naročito u manjim, mukusnim žlezdama. Ponekad mogu da probiju samu kapsulu i da daju izgled formiranja novog tumora, koji se prezentuje kao satelitski nodus; međutim, uvek je u kontaktu sa tumorom. Spoljašnja površina tumora je lobulirana, a na preseku su homogenog izgleda, uglavnom belo prebojeni. Kada je reč o tumoru sa obilnom hrskavičavom ili miksohondroidnom stromom, imaju sedefasti sjaj.

Introduction

Salivary gland tumors are extremely rare neoplasms. Given their pathological image, these tumors represent a great diagnostic challenge. The incidence of these tumors ranges from 3-6% of all tumors of the neck and head region [1,2]. The parotid gland is the most common site of salivary tumor glands, with the incidence of 80-85%, and almost 75% of these tumors are benign neoplasms. The second most frequent site is the submandibular gland with 10%, but one half of tumors of this localization is malignant neoplasms. The sublingual gland is affected in 1% of cases, however, about 80% of tumors are malignant. Minor salivary glands are the sites with the highest incidence of malignant tumors [3,4]. Epidemiological data show a different incidence in different ethnic groups and parts of the world, which further complicates the global incidence of these tumors [5,6]. The mean age of patients is 46-47 years, but the peak of some tumors is in the sixth and seventh decade of life [2].

Pleomorphic adenoma, also referred to as mixed tumor (tumor mixtus), is the most common salivary gland neoplasm. It occurs at all ages, but the peak of incidence is in the fifth decade of life. It is equally represented among the sexes, with a slight domination in women. In 80% of cases, it is localized in the lower pole of the parotid gland. If localized in the deep cortex, it has a presentation of parapharyngeal tumor; they are usually of slow growth and painless. Minor tumors are presented as smooth, solid, mobile globules, whereas large tumors can damage the skin and mucosa which covers them. Multifocal and recurrent tumors may be attached to the surrounding tissue. They may sporadically appear with other tumors, especially with Warthin's tumor. The tumor size varies from 2 to 5 cm, however, they may reach enormous diameters as well. Pain and paresthesia may occur in cases of tumor incarceration. When it comes to the palate, they occur usually on the transition between the soft and hard palate, when they can be attached, due to the proximity of periosteum [7,8].

They are clearly defined macroscopically and have a round to oval shape. The capsule thickness varies, or even lacks, especially in smaller, mucous glands. Sometimes, they can break the capsule itself and appear as if a new tumor was forming, which is presented as a

Patohistološki, tumor je izgrađen od epitelne, mioepitelne i mezenhimne komponente, koja može biti mukoidnog, miksoidnog ili hondroidnog izgleda. Epitelna komponenta formira plaže ili strukture nalik duktusima, a same ćelije mogu biti kuboidalne, vretenaste, plazmocitoidne, skvamozne i ćelije svetle citoplazme. Ponekad epitelna komponenta može biti predominantna u tumoru, što je označeno kao celularni pleomorfni adenom, ali je bez prognostičkog značaja. Duktuse čine luminalne, kuboidalne ćelije, a mogu da imaju i abluminalni sloj mioepitelnih ćelija. Luminalne ćelije, ponekad, imaju svetlu citoplazmu i hiperhromna jedra, što može da zada veliki diferencijalno-dijagnostički problem ka adenoidno-cističnom ili epitelno-mioepitelnom karcinomu. Mezenhimna komponenta tumora je mukoidna, miksoidna, kartilaginozna ili hijalina. i ona može da čini dominantnu komponentu. Ćelije sa mukoidnom supstancom su zapravo mioepitelne ćelije. Ovde mogu da se vide i područja koštane metaplazije. Višegodišnji tumori pokazuju izraženu hijalinizaciju, u tolikoj meri da je epitelna komponenta prisutna u tragovima i sa znacima degeneracije. Takvi tumori predstavljaju veliki rizik za malignu transformaciju [8-10].

Imunohistohemijski, epitelne duktalne ćelije su pozitivne na EMA, CEA GFAP, CK14, dok je mioepitelna komponenta pozitivna na αSMA, p63, vimentin, S100 i GFAP. Poslednjih godina je uočena pozitivna reakcija sa WT1. Naime, modifikovane mioepitelne ćelije pokazuju izrazitu citoplazmatsku ekspresiju proteina [7].

Adenom bazalnih ćelija predstavlja retku benignu neoplazmu pljuvačnih žlezda, bazaloidnog fenotipa. Generalno, sreće se u 1-3% slučajeva svih tumora pljuvačnih žlezda, sa pikom u sedmoj deceniji. Među polovima je češći kod žena, sa odnosom 2:1. Najčešće je lokalizovan u velikim pljuvačnim žlezdama, i to u parotidnoj. Klinički se prezentuje kao jasno ograničeni, pokretni nodus, čvrste konzistencije [7,11,12].

Makroskopski, prezentuju se kao tumori sa kapsulom, solidne do cistične građe, belosive do braon prebojenosti, veličine 1-3 cm. Membranozni tip može biti multinodularan ili multifokalan.

Patohistološki, tumor je izgrađen od bazaloidnih ćelija, nejasnih međućelijskih granica, svetle citoplazme sa ovalnim do okruglim jedrima. Ćelije formiraju solidne, trabekularne ili tubularne formacije.

satellite node. However, it is always in contact with the tumor. The outer surface of the tumor is lobular, it is homogenous at the intersection, and mostly white in color. When it comes to tumors with abundant cartilaginous or mixochondroid stroma, they have a pearly gloss.

Pathohistologically, the tumor is comprised of the epithelial, myoepithelial and mesenchymal component which can have mucoid, myxoid or chondroid appearance. The epithelial component forms nests or duct-like structures, and the cells themselves can be cuboidal, spindle-shaped, plasmocytoid, squamous or light cytoplasm cells. The epithelial component can sometimes be predominant in tumors, which are in such cases labelled as cellular pleomorphic adenomas, but it has no prognostic significance. Ducts are comprised of luminal, cuboidal cells and they can also have the abluminal layer of myoepithelial cells. Luminal cells sometimes have light cytoplasm and hyperchromic nuclei, which can be a great problem in the differential diagnosis of adenoid cystic or epithelial-myoepithelial carcinoma. The mesenchymal component of the tumor is mucoid, myxoid, cartilaginous or hyaline, and it can be the dominant component as well. Cells with mucoid substance are actually myoepithelial cells. Areas of bone metaplasia can also be seen here. Perennial tumors show a pronounced hyalinization, such that the epithelial component is present in traces and with signs of degeneration. Such tumors represent a great risk for malignant transformation [8-10].

Immunohistochemically, ductal epithelial cells are positive to EMA, CEA GFAP, CK14, whereas the myoepithelial component is positive to αSMA, p63, vimentin, S100 and GFAP. A positive reaction with WT1 has been noticed in recent years. Namely, modified epithelial cells exhibit a pronounced cytoplasmic expression of proteins [7].

Basal cell adenoma is a rare benign salivary gland neoplasm of basaloid phenotype. In general, it is found in 1-3% of cases of all salivary gland tumors, with the peak in the seventh decade of life. It is more frequent in women, with a ratio of 2:1. It is usually localized in large salivary glands, especially in the parotid gland. In terms of clinical presentation, it is a clearly circumscribed, mobile node of solid consistency [7,11,12].

Macroscopically, they are presented as encapsulated tumors, of solid to cystic structuire, white-gray to brown-colored, 1-3 cm in size. The membranous type can be multinodular or multifocal.

Kod solidnog tipa rasta, tumorske plaže su različitog oblika sa perifernim radijalnim ćelijskim rasporedom (eng. palisading), međusobno odvojene gustim snopovima kolagenih vlakana. Trabekularni tip se odlikuje bazaloidnim ćelijama koje formiraju uske trake ili trabekule, odvojene vaskularnom stromom. Kod tubularnog tipa dominiraju duktalne strukture. Membranozni tip se karakteriše širokim trakama hijalinog veziva, po periferiji tumorskih plaža, kao i intracitoplazmatskim inkluzijama.

Imunohistohemijski, ćelije na periferiji tumorskih plaža eksprimuju p63, vimentin, SMA i S100, dok su luminalne ćelije pozitivne na CK14, CEA i S100. Analizirajući ekspresiju markera, dolazimo do zaključka da je adenom bazalnih ćelija, histogenetski, poreklom od ćelija interkalatnih kanala [7].

U svakoj varijanti adenoma mogu se videti cistične strukture, skvamozna i onkocitna (u tubularnom tipu) diferencijacija, kao i kribriformni rast [7,13,14].

Polimorfni adenokarcinom niskog gradusa predstavlja primarni maligni epitelni tumor pljuvačnih žlezda sa veoma polimorfnom prezentacijom, monomorfnim ćelijama, infiltrativnim rastom i niskim metastatskim potencijalom. Učestalost tumora iznosi 26% svih intraoralnih karcinoma. Zastupljeniji je u nešto starijoj poulaciji (oko 70% bolesnika je od 50 do 70 godina starosti), sa predominacijom kod žena, i to u odnosu 2:1. U oko 60% slučajeva je lokalizovan na nepcu, potom na bukalnoj sluzokoži, retromolarno, gornjoj usni i podu jezika. Retko je prisutan u velikim pljuvačnim žlezdama. Prezentuje se u vidu bezbolne mase, koja može da bude praćena krvarenjem, telangiektazijama i ulceracijama [7,13].

Makroskopski, tumor je jasno ograničen, ali bez kapsule. Čvrste je konzistencije, prljavo žute prebojenosti, lobuliranog izgleda.

Patohistološki, tumor je izgrađen od monomorfnih ćelija, male do srednje veličine sa malim, ovalnim i hiperhromnim jedrima, baz jasno uočljivih jedaraca. Mitoze i nekroze su retke. Pod polimorfizmom u samom nazivu ovog tumora podrazumeva se solidni, kribriformni, tubularni, trabekularni, fascikularni (eng. streaming), linearni (eng. indian file) i cistični tip rasta. Uočava se targetoidna perineuralna i perivaskularna invazija [7,15].

Stroma može da bude hijalinizovana, mukoidna ili fibrozna, što može dodatno komlikovati diferencijalnu dijagnozu.

Pathohistologically, the tumor is composed of the basaloid cells of blurry intercellular boundaries, with light cytoplasm with oval to round nuclei. The cells form solid, trabecular or tubular formations. In the solid growth type, tumor nests are of different shape with peripheral radial cell arrangement (palisading), separated from each other by thick bundles of collagen fibers. The trabecular type is characterized by the basaloid cells which form narrow strips or trabeculae, separated by the vascular stroma. In the tubular type, ductal structures are dominant. The membranous type is characterized by wide strips of hyaline binder at the periphery of tumor nests, as well as intracytoplasmic inclusions.

Immunohistochemically, the cells at the periphery of tumor beaches express p63, vimentin, SMA and S100, whereas luminal cells are positive to CK14, CEA and S100. Having analyzed the expression of markers, we concluded that basal cell adenoma was histogenetically of intercalated channel cells origin [7].

Cystic structures, squamous and oncocytic (in the tubular type) differentiation, as well as the cribriform growth, can be seen in each variant of adenoma [7,13,14].

Polymorphous low-grade adenocarcinoma is the primary malignant epithelial tumor of the salivary glands, with a rather polymorphous presentation, monomorphic cells, the infiltrative growth and a low metastatic potential. The incidence of the tumor is 26% of all intraoral carcinomas. It is more common in elderly population (around 70% of patients is 50-70 years of age), with a predominance in women with a 2:1 ratio. In about 60% of cases, it is localized on the palate, then on the buccal mucosa, in the retromolar area, on the upper lip and the floor of the tongue. It is rarely found in large salivary glands. It is presented in the form of a painless mass which may be accompanied by bleeding, telangiectasia and ulceration [7, 13].

Macroscopically, the tumor has clear boundaries, but without a capsule. It is of firm consistency, dirty-yellow in color, and of a lobulated appearance.

Pathohistologically, the tumor is composed of the monomorphic cells, of small to medium size, with small, oval and hyperchromatic nuclei, but without clearly visible nucleoli. Mitosis and necrosis are rare. The term polymorphous in the name of this tumor stands for a solid, cribriform, tubular, trabecular, fascicular (streaming), linear (indian file) and cystic growth type.

Imunohistohemijski, tumorske ćelije su pozitivne na CEA, EMA, vimentin, S100 i CK14, a negativne na p63, αSMA i GFAP [7].

Diferencijalno dijagnostički se uključuje pleomorfni adenom i adenoidni cistični karcinom.

Cilj

S obzirom na polimorfizam i preklapanje mikromorfološke prezentacije pleomorfnog adenoma, adenoma bazalnih ćelija i polimorfnog adenokarcinoma niskog gradusa, cilj našeg istaživanja bio je diferencijacija tri tipa tumora primenom imunohistohemijske i morfometrijske analize, kao i određivanje visine Ki67 proliferativnog indeksa.

Materijal i metode

Celokupno istraživanje je sprovedeno na Institutu za patologiju Medicinskog fakulteta u Nišu. Analizirani materijal predstavlja tkivo dobijeno operacijom i biopsijom pljuvačnih žlezda, sa Klinike za Maksilofacijalnu hirurgiju u Nišu. Odnos polova je varirao u zavisnosti od tumora, ali je prisutna blaga predominacija ženskog pola, što je izraženije sa malignim tumorima. Starost bolesnika varirala je u opsegu od 12 do 75 godina, naročito kod benignih lezija. Maligniteti u dečijem dobu nisu bili registrovani. Nakon primenjene intervencije, tkivo se fiksira u 10% formalinu, najmanje 24 h, po preporukama Američkog udruženja onkologa i koledža patologa (*eng. American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP))* [16]. Istraživanje je obuhvatilo 44 reprezentativna tumora pljuvačnih žlezda, 20 pleomorfnih adenoma, 12 adenoma bazalnih ćelija i 12 polimorfnih adenokarcinoma niskog gradusa. Od ukupno 44 pregledana materijala, 40 je bilo operativnih, a četiri su biopsijskih materijala. Detaljniji prikaz analiziranih tumora dat je u Tabeli 1. Mikroskopskom analizom dobijenih preparata vršena je preliminarna dijagnostika. Istovremeno, odeređivan je i reprezentativni isečak, što podrazumeva veličinu tumorskog polja sa minimalnim poljima nekroze i zapaljenskim infiltratom na kome su se radila imunohistohemijska bojenja. Imajući u vidu polimorfnost tumora pljuvačnih žlezda, još jedan od parametara za izbor isečka bila je i najpolimorfnija slika na patohistološkom preparatu. Kao kontrola služilo je zdravo tkivo pljuvačne žlezde.

Targetoid perineural and perivascular invasion can be seen [7, 15].

The stroma can be hyalinized, mucoid or fibrous, which can further complicate the differential diagnosis.

Immunohistochemically, tumor cells are positive to CEA, EMA, vimentin, S100 and CK14, but negative to p63, αSMA and GFAP [7].

The differential diagnosis includes pleomorphic adenoma and adenoid cystic carcinoma.

Aim

Given the polymorphism and overlapping of the micromorphological presentation of pleomorphic adenoma, basal cell adenoma and polymorphous low-grade adenocarcinoma, the aim of our study was to differentiate these three tumor types by applying the immunohistochemical and morphometric analysis.

Material and Methods

The entire study was conducted in the Center for Pathology and Pathological Anatomy in Niš. The analyzed material was a tissue obtained by the surgical procedure or biopsy of salivary glands, from the Maxillofacial Surgery Clinic in Niš. The gender ratio varied depending on the tumor, but there was a slight predominance of women, especially in cases of malignant tumors. The age of the patients also varied, ranging from 12 to 75 years, especially in benign lesions. Malignity in children was not registered. Upon the applied intervention, the tissue was fixed in 10% formalin, as recommended by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [16]. The study included 44 representative salivary gland tumors, 20 pleomorphic adenomas, 12 basal cells adenomas and 12 polymorphous low-grade adenocarcinomas. Forty out of the total of 44 examined specimens were operational, whereas the remaining 4 were biopsy materials. A more detailed review of the analyzed tumors is presented in Table 1. The preliminary diagnostics included the microscopic analysis of the obtained preparations. Simultaneously, a representative tissue sample was determined, i.e. part of the tumor area with minimal necrosis and inflammatory infiltrate on which immunohistochemical staining had been done.

Analizirana je ekspresija Ki67, p53 i HER-2 antigena, kao marker proliferacije. U sklopu diferencijalne dijagnostike analizirana je ekspresija karcino-embrionalnog antigena, epitelnog membranskog antigena, kiselog glijalnog fibrilarnog proteina, p63 antigena, vimentina, citokeratina 14, α-glatkomišićnog aktina, S-100 protein i Vilms tumor 1 antigena. Pravljene su digitalne mikrofotografije, kako osnovnih tako i preparata dobijenih imuno-histohemijskim bojenjem.

Imunohistohemijska bojenja vršena su na isečcima debljine do 5μm, dobijenih iz parafinskih blokova. Za analizu je uziman reprezentativan isečak, a primenjena su sledeća antitela: anti-Ki67 (MiB-1, ready to use; DAKO, Glostrup, Denmark), anti-p53 (DO-7, ready to use; DAKO, Glostrup, Denmark), anti-p63 (e DAK-p63, ready to use; DAKO, Glostrup, Denmark), anti-HER-2 (HercepTest™, DAKO, Glostrup, Denmark), anti-CEA (II-7, ready to use; DAKO, Glostrup, Denmark), anti-EMA (E29, ready to use; DAKO, Glostrup, Denmark), anti-S-100 (S-100, ready to use; DAKO, Glostrup, Denmark), anti-CK14 (LL002, 1:20; Novocastra Laboratories, Newcastle, UK), anti-WT-1 (6F-H2, ready to use; DAKO, Glostrup, Denmark), anti-GFAP (6F2, ready to use; DAKO, Glostrup, Denmark), anti-αSMA (1A4, ready to use; DAKO, Glostrup, Denmark), anti-vimentin (V9, ready to use; DAKO, Glostrup, Denmark).

Za pozitivnu reakciju smatra se bojenje jedara za sledeće markere: Ki67, p53, p63 i WT1. Pozitivno membransko bojenje analizirano je za HER-2, a za CEA i EMA membransko i citoplazmatsko, a za S-100 jedarno i citoplazmatsko. Pozitivno citoplazmatsko bojenje vidi se kod αSMA, GFAP, CK14 i vimentina.

Ki67 indeks određivan je analizom i brojanjem pozitivnih jedara na 10 vidnih polja, na uveličanju x40. Indeks je izražen u procentima, kao odnos pozitivnih tumorskih ćelija u odnosu na negativne, neobojene ćelije.

Morfometrijska analiza vršena je u softverkom paketu „*ImageJ*" verzija 1.43u (public domain software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). Mikrofotografije u boji dobijene su digitalnom kamerom visoke rezolucije (Nikon, DS-Fi1, Tokyo, Japan), koja je povezana sa mikroskopom (Nikon, ECLIPSE 50i, Tokyo, Japan). Nakon toga, slika je prebačena na kompatibilni računar i vršena je analiza jedarnih parametara primenom paketa.

Considering the polymorphism of salivary gland tumors, one of the parameters for the selection of tissue samples was the most polymorphous image on the patho-histological preparation. The healthy tissue of the salivary gland served as the control.

We analyzed the expression of Ki67, p53 and HER-2 antigens, as well as the expression of proliferation makers. The expression of the carcinoembryonic antigen, epithelial membrane antigen, glial fibrillary acidic protein, p63 antigen, vimentin, cytokeratin 14, α-smooth muscle actin, S-100 protein and Wilms' tumor 1 antigen was analyzed within the differential diagnosis. Digital photomicrographs of both basic and preparations obtained from immuno-histochemical staining were made.

Immunohistochemical staining was done on tissue samples of up to 5μm of thickness, obtained from paraffin blocks. A representative tissue sample was taken for the analysis, and the following antibodies were applied: anti-Ki67 (MiB-1, ready to use; DAKO, Glostrup, Denmark), anti-p53 (DO-7, ready to use; DAKO, Glostrup, Denmark), anti-p63 (e DAK-p63, ready to use; DAKO, Glostrup, Denmark), anti-HER-2 (HercepTest™, DAKO, Glostrup, Denmark), anti-CEA (II-7, ready to use; DAKO, Glostrup, Denmark), anti-EMA (E29, ready to use; DAKO, Glostrup, Denmark), anti-S-100 (S-100, ready to use; DAKO, Glostrup, Denmark), anti-CK14 (LL002, 1:20; Novocastra Laboratories, Newcastle, UK), anti-WT-1 (6F-H2, ready to use; DAKO, Glostrup, Denmark), anti-GFAP (6F2, ready to use; DAKO, Glostrup, Denmark), anti-αSMA (1A4, ready to use; DAKO, Glostrup, Denmark), anti-vimentin (V9, ready to use; DAKO, Glostrup, Denmark).

The staining of nuclei was considered positive for the following markers: Ki67, p53, p63 and WT1. Positive membrane staining was analyzed for HER-2, membrane and cytoplasmic staining for CEA and EMA, whereas nuclear and cytoplasmic staining was analyzed for S-100. Positive cytoplasmic staining could be seen in αSMA, GFAP, SK14 and vimentin.

The Ki67 index was determined by analyzing and counting the positive nuclei in 10 visible areas, at the magnification of x40. The index was expressed in percentage, as the ratio between positive tumor cells and negative, unstained cells. The morphometric analysis was done in the software pack "*ImageJ*" version 1.43 u (public domain

Osmobitna slika je manuelno obrađivana, nakon kalibracije, korišćenjem kompjuterskog miša. Analizirano je 100 nasumično odabranih tumorsko-ćelijskih jedara, na uveličanju x40, i to ćelija koje se ne preklapaju. Analizirano je šest jedarnih parametara: površina (eng. area), perimetar (*eng. perimeter*), cirkularnost (eng. circularity), zaobljenost (*eng. roundness*), Feretov dijametar (*eng. Feret diameter*) i integrisana optička gustina (*eng. Integrated Optical Density).*

Statistička obrada podataka

Statistička analiza podataka rađena je u programskom paketu SPSS 15.0. Dobijeni rezultati su prikazani tabelarno.

Kontinualne varijable su predstavljene osnovnim statističkim parametrima – aritmetičkom sredinom ($\overline{X}$), standardnom devijacijom (SD), medijanom (Me) kao merom centralne tendencije, te opsegom, tj. minimalnim i maksimalnim vrednostima. Kvalitativna obeležja ispitivanih promenljivih data su učestalošću (n) i procentualnom zastupljenošću (%).

U zavisnosti od veličine uzorka, normalnost distribucije kontinualnih varijabli, ispitivana je Kolmogorov-Smirnov ili Shapiro-Wilkovim testom.

Za ocenu značajnosti razlike (p) kontinualnih varijabli između dve nezavisne grupe ispitanika korišćeni su Studentov t-test nezavisnih uzoraka, kod normalne distribucije podataka, ili Mann-Whitnijev U test, kod distribucije koja odstupa od normalne. Kao prag statističke značajnosti definisana je standardna vrednost, $p < 0,05$.

Za testiranje značajnosti razlike između više nezavisnih grupa korišćena je ANOVA, a na osnovu testiranja homogenosti varijansi po Levenu sprovedena je sledstvena Post Hoc analiza, odnosno multipna poređenja Tukey HSD (za homogene varijanse) ili Tamhaneovim testom u slučaju nehomogenosti varijansi.

Za testiranje statističke značajnosti razlika apsolutnih frekvencija između uzoraka korišćen je χ^2 test, ili Fisherov test egzaktne verovatnoće, ukoliko je apsolutna frekvencija obeležja manja od 5.

software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). Photomicrographs, in colour, were obtained using a high-resolution digital camera (Nikon, DS-Fil, Tokyo, Japan), which was connected to the microscope (Nikon, ECLIPLE 50i, Tokyo, Japan). After that, the picture was transferred to the compatible computer and the analysis of nuclear parameters was carried out using the pack. The eight-bit picture was manually processed after the calibration, using a computer mouse. We analyzed 100 random tumor cell nuclei, at the magnification of x40, i.e. cells that did not overlap. We also analyzed six nuclear parameters: area, perimeter, circularity, roundness, Feret diam-eter and integrated optical density.

Statistical data analysis

The statistical analysis of data was performed in the program pack SPSS 15.0. The obtained results are presented in tables.

Continuous variables are presented by basic statistical parameters: mean value ($\overline{X}$), standard deviation (SD), median (Me) as a measure of central tendency, range, i.e. minimal and maximal values. Quantitative characteristics of the examined variables were determined based on frequency (n) and the percentage share (%).

Depending on the size of the sample, the normality of the distribution of continuous variables was examined using the Kolmogrov-Smirnov or Shapiro-Wilk test.

To evaluate the significance of the difference (p) of continuous variables between two independent groups of subjects, the Student's t-test of independent samples was used in case of the normal distribution of data, i.e. the Mann-Whitney U test in case of the distribution which deviates from the normal one. The standard value $p < 0.05$ was defined as the threshold of statistical significance.

ANOVA was used to test the significance of the difference between several independent groups, and based on the Levene's test for the homogeneity of variances, a sequential post-hock analysis was carried out, i.e. multiple comparisons using the Tukey's HSD (for homogenous variances) or the Tamhane's test in cases of non-homogenous variances.

To test the statistical significance of differences in absolute frequencies between samples, we used the χ^2 test, or the Fisher's exact probability test, if the absolute frequency of a sample was less than 5.

Rezultati

Na osnovu već iznetih mikromorfoloških karakteristika, analizirano je ukupno 44 tumora pljuvačnih žlezda, i to 20 pleomorfnih adenoma (Slika 1), 12 adenoma bazalnih ćelija (Slika 2) i 12 polimorfnih adenokarcinoma niskog gradusa (Slika 3 i 4).

Rezultati imunohistohemijske analize ekspresije markera proliferacije i intermedijarnih filamenata, kao i ostalih markera koji su analizirani u istraživanju prikazani su u Tabeli 2.

Ekspresija proteina se prati u duktalnoj i mioepitelnoj komponenti pleomorfnog adenoma. Neoplastične ćelije su pokazale jaku ekspresiju GFAP (20/20), p63 (20/20), WT1 (20/20), vimentin (18/20) i S100 (16/20) (Slika 5).

U grupi adenoma bazalnih ćelija analizirali smo sva četiri tipa rasta tumora. Ukupno je bilo dvanaest slučajeva iz ove grupe tumora. Najbrojniji je, generalno, solidni tip rasta. Praćena je ekspresija u luminalnim i bazaloidnim ćelijama. Odsustvo pozitivne reakcije primećeno je za GFAP. WT1, CEA i S100 su pokazali fokalnu pozitivnost, vimentin i CK14 su se eksprimirali u bazaloidnim ćelijama u tubularnom tipu, odnosno na periferiji tumorskih plaža u solidnom tipu rasta (Slika 6).

U grupi od dvanaest polimorfnih adenokarcinoma niskog gradusa prisutna je difuzna ekspresija CK14 (12/12). S100, vimentin i EMA su bili apsolutno eksprimirani, dok je αSMA bio negativan. Uočena je fokalna ekspresija WT1, naročito u kribriformnim delovima tumora (Slika 7).

Analizom vrednosti proliferativnog Ki67 indeksa ustanovljena je statistički značajna razlika u grupi pleomorfnog adenoma (Tumor mixtus), što se dovodi u vezu sa čestim recidiviranjem ovog benignog tumora.

Rezultati analize morfometrijskih karakteristika

Analizom morfometrijskih karakteristika uočavaju se veće vrednosti u grupi polimorfnog adenokarcinoma niskog gradusa, ali statistički značajne razlike nađene su samo za Feretov dijametar i integrisanu optičku gustinu ($p < 0,05$), kao i veće vrednosti za integrisanu optičku gustinu u grupi adenoma bazalnih ćelija ($p < 0,001$)

Results

Based on the already outlined micromorphological characteristics, we analyzed a total of 44 salivary gland tumors – 20 pleomorphic adenomas (*Figure 1*), 12 basal cell adenomas (*Figure 2*), and 12 polymorphous low-grade adenocarcinomas (*Figure 3 and 4*).

The results of immunohistochemical analysis of the expression of proliferation markers and intermediary filaments, as well as other markers analyzed in the study, are shown in Table 2.

The expression of proteins was monitored in the ductal and myoepithelial component of pleomorphic adenoma. Neoplastic cells showed a strong expression of GFAP (20/20), p63 (20/20), WT1 (20/20), vimentin (18/20) and S100 (16/20) (Figure 5).

We analyzed all four types of tumor growth in the basal cell adenoma group. Twelve cases from this tumor group were present. In general, the most common was the solid tumor growth. The expression in luminal and basal cells was also monitored. The absence of a positive reaction was noticed for GFAP. WTI, CEA, S100 showed focal positivity, vimentin and CK14 were expressed in basaloid cells in the tubular type, i.e. at the periphery of tumor nests in the solid growth type (Figure 6).

Diffuse expression of CK14 (12/12) was present in the group of 12 polymorphous low-grade adenocarcinomas. S100, vimentin and EMA were absolutely expressed, whereas αSMA was negative. Focal expression of WT1 was noticed, especially in the cribriform parts of the tumor (Figure 7).

The analysis of the proliferative Ki67 index values showed a statistically significant difference in the pleomorphic adenoma group (Tumor mixtus), which was associated with frequent recurrence of this benign tumor.

Results of the analysis of morphometric characteristics

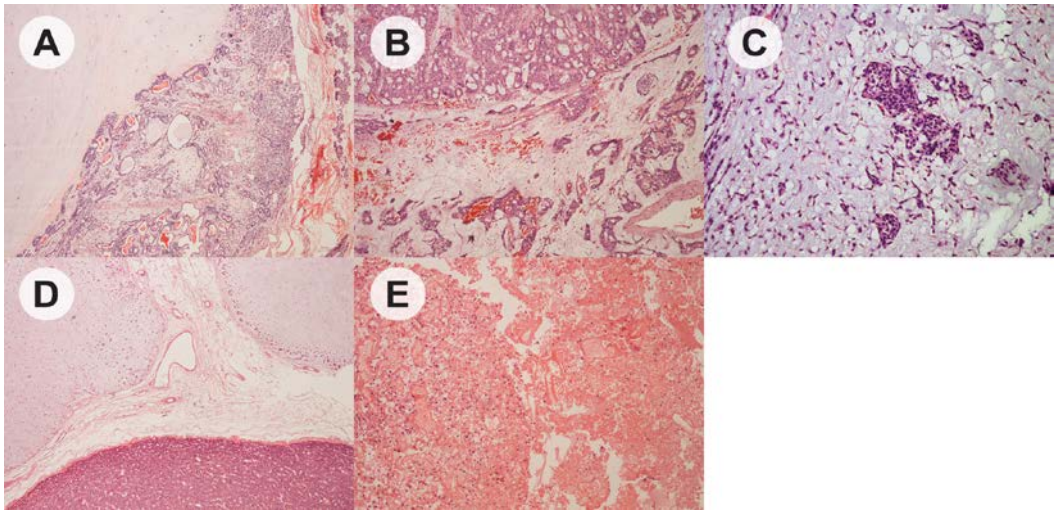
The analysis of morphometric characteristics showed higher values in the polymorphous low-grade adenocarcinoma group, but statistically significant differences were found only for Feret diameter and integrated optical density ($p < 0.05$), as well as higher values for integrated optical density in the basal cell adenoma group ($p < 0.001$).

Diskusija

Velika dijagnostička dilema može da nastane u diferencijaciji pleomorfnog adenoma i polimorfnog adenokarcinoma niskog gradusa. To je naročiti problem kada se govori o malim, incizionim biopsijama. U tom slučaju, može se stvoriti lažna slika o infiltrativnom rastu, ako je reč o tumorima malih pljuvačnih žlezda, gde pleomorfni adenom najčešće ne poseduje kapsulu, a može imati i fokalne ekstenzije u susedne žlezde i prezentovati se kao maligna neoplazma. Oba tipa tumora se sastoje od relativno uniformnih ćelija koje karakteriše odsustvo atipije [17]. Veoma bitan parametar o malignitetu, kada je reč o polimorfnom adeno-karcinomu niskog stepena, jeste perineuralna invazija, o kojoj se ne može izjašnjavati na malom biopsijskom uzorku.

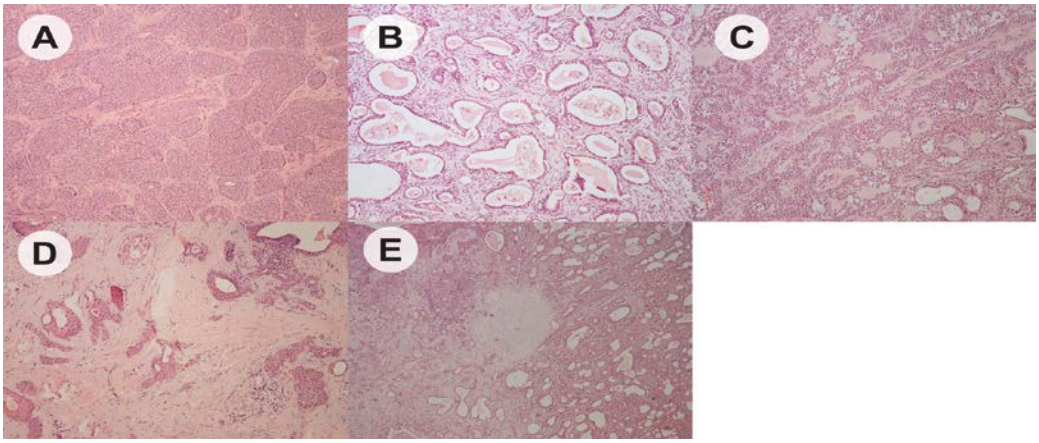
Discussion

A great diagnostic dilemma may arise in the differentiation of pleomorphic adenoma and polymorphous low-grade adenocarcinoma. It may be a problem especially when it comes to small, incisional biopsies. It that case, a false picture about the infiltrative growth may be created regarding minor salivary gland tumors where pleomorphic adenoma does not possess the capsule, but may have focal extensions in the neighboring glands and present itself as a malignant neoplasm. Both tumor types consist of relatively uniform cells which are characterized by the absence of atypia [17]. When it comes to polymorphous low-grade carcinoma, the perineural invasion is a very important parameter for malignity, and it cannot be determined based on a small biopsy sample.



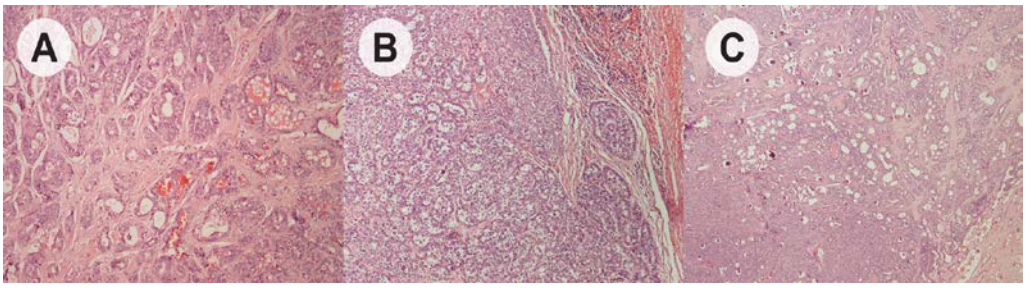
***Slika 1*. Pleomorfni adenom**, **A** – epitelna komponenta tumora formira solidno-cistične plaže sa hondroidnom stromom, H&E, uveličanje x4; **B** – celularni tip tumora sa tubularnom epitelnom komponentom, H&E, uveličanje x10; **C** – dominanta mukoidna stroma tumora, H&E, uveličanje x20; **D** – sinhroni rast pleomorfnog adenoma i mioepitelioma, H&E, uveličanje x4; **E** – područje ishemijske nekroze, H&E, uveličanje x10

***Figure 1*. Pleomorphic adenoma; A –** epithelial tumor component forms solid-cystic nests with chrondroid stroma, H&E, magnification x4; B – cellular tumor type with tubular epithelial component, H&E, magnification x10; **C** - dominant mucoid tumor stroma, H&E, magnification x20; **D** – synchronous growth of pleomorphic adenoma and myoepithelioma, H&E, magnification x4; **E** – area of ischemic necrosis, H&E, magnification x10;



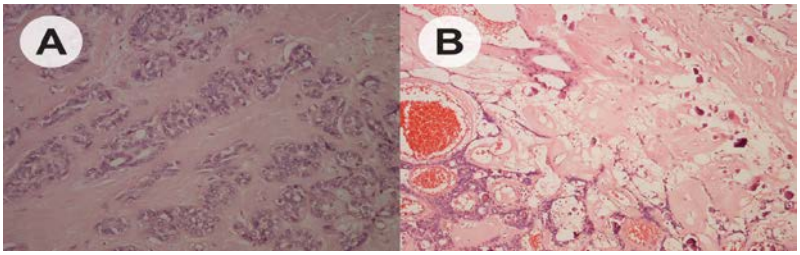
***Slika 2*. Adenom bazalnih ćelija**; **A** – solidni tip rasta tumora, H&E, uveličanje x10; **B** - tubularni tip rasta tumora, H&E, uveličanje x10; **C** - trabekularni tip rasta tumora, H&E, uveličanje x10; **D** - membranozni tip rasta tumora, H&E, uveličanje x10; **E** - tubularni tip rasta tumora sa područjem hondroidne metaplazije, H&E, uveličanje x10

***Figure 2*. Basal cell adenoma**; **A** – solid tumor growth type, H&E, magnification x10; **B** - tubular tumor growth type, H&E, magnification x10; **C** - trabecular tumor growth type, H&E, magnification x10; **D** - membranous tumor growth type, H&E, magnification x10; **E** - solid tubular growth type with area of chondroid metaplasia, H&E, magnification x10;



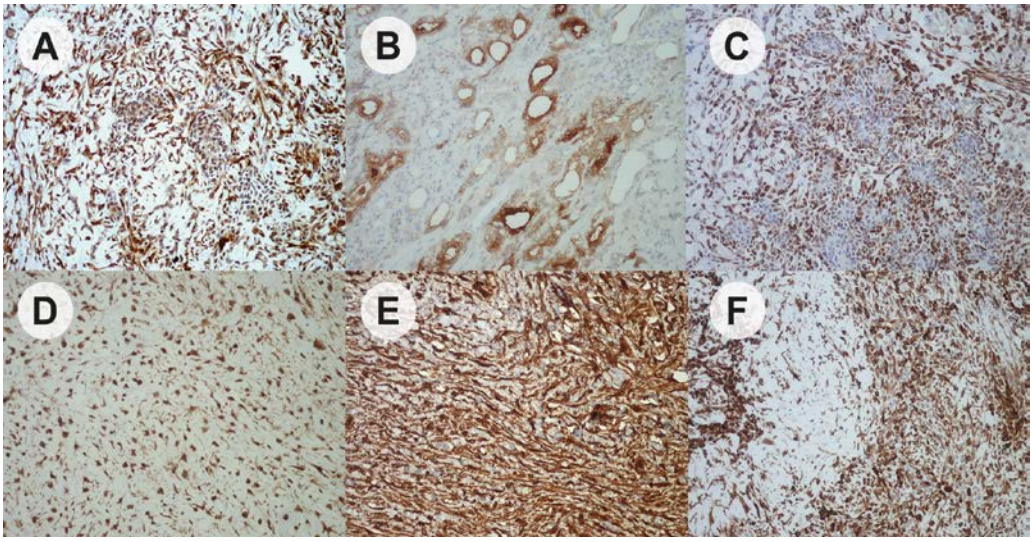
***Slika 3*. Polimorfni adenokarcinom niskog gradusa**, **A** – tubularni tip rasta, H&E, uveličanje x10; **B** – solidno-kribriformni tip rasta, H&E, uveličanje x10, **C** – solidno-cistični tip rasta, H&E, uveličanje x4

***Figure 3*. Polymorphous low-grade adenocarcinoma**; **A** – tubular growth type, H&E, magnification x10; **B** – solid-cribriform growth type, H&E, magnification x10, **C** – solid-cystic growth type, H&E, magnification x4;



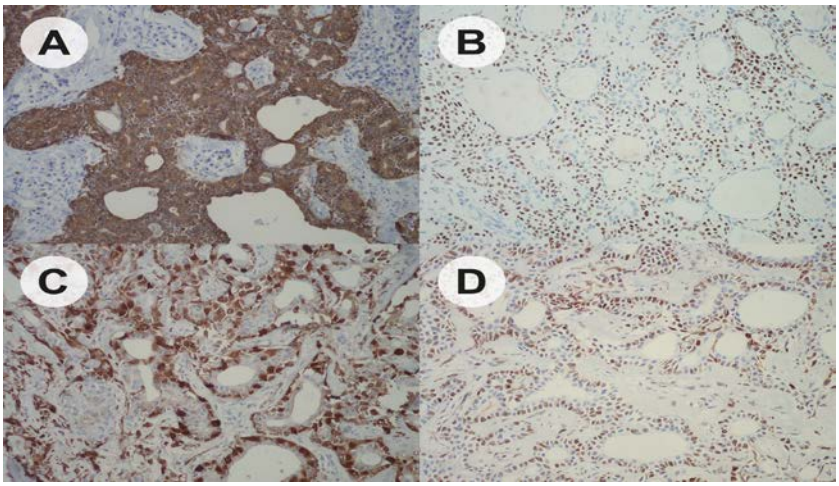
***Slika 4*. Polimorfni adenokarcinom niskog gradusa**, **A** – tubularni tip rasta tumora sa hijalinizovanom stromom, H&E, uveličanje x10; **B** – kribriformni tip rasta tumora sa mukoidnom stromom i mikrokalcifikatima, H&E, uveličanje x10

***Figure 4*. Polymorphous low-grade adenocarcinoma**; **A** – tubular growth type with hyalinized stroma, H&E, magnification x10; **B** – cribriform growth type with mucoid stroma and microcalcifications, H&E, magnification x10;



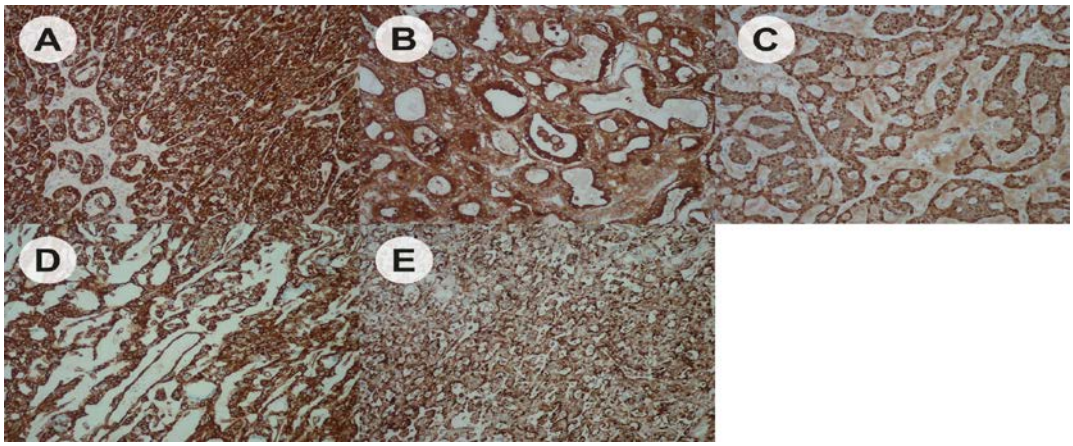
Slika 5. Tumor mixtus, uveličanje x20; **A** – Difuzna, intenzivna ekspresija αSMA u mioepitelnoj komponenti tumora; **B** – Ekspresija CEA u luminalnim ćelijama; **C** – intenzivna citoplazmatska ekspresija GFAP u epitelnim i mioepitelnim ćelijama; **D** – Jedarna ekspresija S100; **E** – Citoplazmatska ekspresija vimentina; **F** – Ekspresija WT1 u citoplazmi neoplastičnih mioepitelnih ćelija

Figure 5. **Tumor mixtus**; magnification x20; **A** – diffuse, intensive expression of αSMA in myopithelial tumor component; **B** – expression of CEA in luminal cells; **C** – intensive cytoplasmic expression of GFAP in epithelial and myoepithelial cells; **D** – nuclear expression of S100; **E** – cytoplasmic expression of vimentin; **F** – expression of WT1 in the cytoplasm of neoplastic myoepithelial cells;



Slika 6. Basal cell adenoma, uveličanje x20; **A** – CK14 – intenzivna citoplazmatska ekspresija u neoplastičnim ćelijama; **B** – Jedarna ekspresija p63 u bazaloidnim ćelijama; **C** – Jedarna i citoplazmatska ekspresija S100 u bazaloidnim ćelijama; **D** – Intenzivna citoplazmatska i slaba jedarna ekspresija WT1 u bazaloidnim ćelijama tumorskih plaža

Figure 6. **Basal cell adenoma**; magnification x20; **A** – CK14 – intensive cytoplasmic expression in neoplastic cells; **B** – nuclear expression of p63 in basaloid cells; **C** – nuclear and cytoplasmic expression of S100 in basaloid cells; **D** – intensive cytoplasmic and weak nuclear expression of WT1 in basaloid cell of tumor nests;



Slika 7. Polymorphus low-grade adenocarcinoma, uveličanje x20; **A** – Difuzna i intenzivna ekspresija CK14; **B** – Ekspresija EMA u luminalnim i abluminalnim ćelijama; **C** – Homogeno prebojavanje neoplastičnih ćelija S100; **D** – Citoplazmatska ekspresija vimentina; **E** – Ekspresija WT1 u kribriformnom delu tumora

Figure 7. **Polymorphous low-grade adenocarcinoma**, magnification x20; **A** – diffuse and intensive expression of CK14; **B** – expression of EMA in luminal and abluminal cells; **C** – homogenous S100 staining of neoplastic cells; **D** – cytoplasmic expression of vimentin; **E** – expression of WT1 in cribriform part of the tumor;

Tabela 1. Karakteristike pacijenata
Table 1. Characteristics of patients

Tumor type Tip tumora	Number (N) Broj (N)	Sex (man/woman) Pol (muškarac/žena)	Mean age Srednja starost
Pleomorphic adenoma Pleomorfni adenom	20	12/8	40
Basal cell adenoma Adenom bazalnih ćelija	12	4/8	67
Polymorphus low-grade adenocarcinoma Polimorfni adenokarcinom niskog gradusa	12	4/8	64

Tabela 2. Imunohistohemijska ekspresija
Table 2. Immunuhistochemical expression

Tumor type Tip tumora	CEA	EMA	Vimentin	p63	αSMA	S100	CK14	WT1	GFAP	p53	HE R2
Tumor mixtus Tumor mixtus	+ (10/20) L	+ (10/20) L	+ (18/20) ABL	+ (20/20) ABL	+ (14/20) ABL	+ (16/20) ABL	+ (14/20) L/ABL	+ (20/20) ABL	+ (20/20) ABL	+ (2/20) L	- (0/2 0)
Basal cell adenoma	+ (6/12) L	+ (8/12) L	+ (12/12) ABL	+ (12/12) ABL	+ (10/12) ABL	+ (8/12) ABL	+ (12/12) ABL	+ (6/12) ABL	- (0/12)	+ (6/12) L/ABL	- (0/1 2)
Polymorphus low-grade adenocarcinoma	+ (10/12) L/ABL	+ (12/12) L/ABL	+ (12/12) ABL	+ (2/12) ABL	- (0/12)	+ (12/12) L/ABL	+ (12/12) L/ABL	+ (4/12) ABL	- (0/12)	+ (6/12) L/ABL	- (0/1 2)

+ prisutna ekspresija proteina; - odsutna ekspresija proteina; ABL – abluminalne ćelije; L – luminalne ćelije
+ present protein expression; - absent protein expression; ABL – abluminal cells; L – luminal cells

Tabela 3. Vrednosti proliferativnog indeksa u odnosu na tip tumora
Table 3. Proliferative index values regarding the tumor type

Tumor type Tip tumora	X ± SD	(Me)	Min –	Max
Tumor mixtus	5,97 ± 4,05 *	(4,60)	1,20 –	13,00
Basal cell adenoma	3,85 ± 3,71	(2,34)	0,76 –	12,00
Polymorphus low grade adenocarcinoma	2,57 ± 1,32	(2,78)	1,18 –	4,78

Podaci su predstavljeni kao X ± SD (Me) Min–Max
* – p < 0,05
Data are presented as X ± SD (Me) Min–Max
* – p < 0,05

Tabela 4. Morfometrijske karakteristike jedara tumorskih ćelija
Table 4. Morphometric characteristics of tumor cell nuclei

		Basal cell adenoma (n = 12)	Polymorphus low-grade adenocarcinoma (n = 12)
Area	35,82 ± 3,57	40,97 ± 7,70	54,23 ± 12,38
Perimeter	23,28 ± 1,37	24,04 ± 2,09	27,85 ± 2,98
Circularity	0,83 ± 0,02	0,87 ± 0,02	0,86 ± 0,02
Feret diameter	8,66 ± 0,49	9,07 ± 0,65	10,42 ± 0,90*
IntDent	4,53 ± 0,38	15,81 ± 4,54 **	18,71 ± 6,79*
Roundness	0,69 ± 0,04	0,71 ± 0,05	0,70 ± 0,05

Podaci su predstavljeni kao X ± SD (Me) Min–Max
* – p < 0,05, ** – p < 0,001 (ANOVA, Post Hoc Test, Tamhane test)

Data are presented as X ± SD (Me) Min–Max
* – p < 0,05, ** – p < 0,001 (ANOVA, Post Hoc Test, Tamhane's test)

U ovakvim slučajevima neophodna je primena imunohistohemije. U prvom redu se primenjuju markeri mioepitelne diferencijacije i GFAP. Nakazato i saradnici su još 1982. godine ukazali na ukrštenu ekspresiju GFAP, vimentina i citokeratina u diferencijaciji pleomorfnog adenoma [18].GFAP pokazuje veoma izraženu citoplazmatsku ekspresiju u duktalnim epitelnim i mioepitelnim ćelijama adenoma, dok je zanemarljivo mala pozitivnost registrovana u ćelijama polimorfnog adenokarcinoma niskog gradusa [19]. Naši rezultati pokazuju potpuno odsustvo pozitivne reakcije GFAP kod ovog tipa karcinoma, a apsolutnu pozitivnost kod pleomorfnog adenoma, što je u skladu sa velikim studijama koje su se bavile diferencijalnom dijagnozom ova dva entiteta [20,21].

In such cases, it is necessary to apply immunohistochemistry. Firstly, markers of myoepithelial differentiation and GFAP are applied. Back in 1982, Nakazato et al. pointed to the cross-over expression of GFAP, vimentin and cytokeratin in the differentiation of pleomorphic adenoma [18] .GFAP exhibited a very complex cytoplasmic expression in ductal epithelial and myoepithelial adenoma cells, whereas insignificant positivity was registered in the cells of polymorphous low-grade adenoma [19]. Our results show a complete absence of the positive reaction of GFAP in this type of carcinoma, and absolute positivity in pleomorphic adenoma, which is an accordance with large studies dealing with the differential diagnosis of these two entities [20,21].

Takođe, veoma značajan nalaz u diferencijaciji jeste i ekspresija αSMA u pleomorfnom adenomu koja je odsutna u polimorfnom adenokarcinomu niskog gradusa [22]. Za dokazivanje neoplastičnih (modifikovanih) mioepitelnih ćelija, u poslednje vreme, koristi se WT1. Leader i Langman su u svojim studijama ukazivali na značaj ovog markera. WT1 je pokazao apsolutnu citoplazmatsku pozitivnost u abluminalnim ćelijama sa mioepitelnom diferencijacijom pleomorfnog adenoma [23]. Te ćelije su pokazale i ekspresiju p63. Kod polimorfnog adenokarcinoma niskog gradusa ekspresija je varirala u zavisnosti od varijante ćelijskog aranžmana. U skladu sa nalazima objavljenih studija, naši rezultati ukazuju na značaj imunohistohemijskog dokazivanja mioepitelnih ćelija u dijagnostici polimorfnog adenokarcinoma niskog gradusa.

Adenom bazalnih ćelija je uveden u klasifikaciju Svetske zdravstvene organizacije 1991. godine. Do tada je označavan kao nepleomorfni adenom ili monomorfni adenom. Na osnovu histološke prezentacije i načina rasta i rasporeda neoplastičnih ćelija, podeljen je u više varijanti, solidni, koji je i najčešći, tubularni, trabekularni i membranozni [15]. Uglavnom se u tumoru viđa više varijanti načina rasta, ali je jedna dominantna, na osnovu koje se dalje vrši morfološka subklasifikacija adenoma bazalnih ćelija. Svi imaju fibroznu stromu, ali bez miksohondroidnih područja, koja se viđaju u pleomorfnom adenomu. Cistična degeneracija, skvamozna metaplazija, keratinizacija i kribriformni rast su povećali mogućnost ovom tumoru za dijagnostičku grešku. Generalno, tumorske plaže su izgrađene od tamnih, plavih ćelija na periferiji, koje često imaju palisadni aranžman. U centralnim delovima su prisutne nešto krupnije ćelije, svetlije citoplazme. Kod svih varijanti postoji mioepitelna diferencijacija, koja na standardnim H&E preparatima nije očigledna, ali imunohistohemijskom analizom je dokazana [24]. U diferencijalnu dijagnozu adenoma bazalnih ćelija se uključuje polimorfni adenokarcinom niskog gradusa i pleomorfni adenom, kao i neke od varijanti mioepitelioma. Imunohistohemijskom analizom naših rezultata, a u skladu sa podacima iz literature, pokazali smo da ćelije na periferiji tumorskih plaža eksprimuju p63, vimentin, SMA i S100, dok su ćelije u unutrašnjosti ili luminalne, ako govorimo o tubularnoj varijanti, pozitivne na CK14, CEA i S100.

Moreover, a very significant finding in the differentiation includes the expression of αSMA in pleomorphic adenoma which is absent in polymorphous low-grade adenocarcinoma [22]. In recent years, WT1 has been used for the determination of neoplastic cells. Leader and Langman emphasized the significance of this marker in their studies. WT1 showed absolute cytoplasmic positivity in abluminal cells with myoepithelial differentiation of pleomorphic adenoma [23]. Those cells exhibited the expression of p69. In polymorphous low-grade adenocarcinoma, the expression varied depending on the variant of cell arrangement. In accordance with the findings of published studies, our results emphasize the importance of immunohistochemical detection of myoepithelial cells in the diagnostics of polymorphous low-grade adenocarcinoma.

Basal cell adenoma was classified by the World Health Organization in 1991. By that time, it had been referred to as non-pleomorphic adenoma or monomorphic adenoma. Based on the histological presentation, growth type and neoplastic cell arrangement, it was divided into several variants: solid, which is the most common, tubular, trabecular and membranous [15]. The tumor usually exhibits many growth type variants, with one of them being dominant, based on which further morphological subclassification of basal cell adenoma is done. They all have the fibrous stroma, but without myxochondroid areas which can be seen in pleomorphic adenoma. Cystic degeneration, squamous metaplasia, keratinization and the cribriform growth have increased the possibility of diagnostic errors for this tumor. In general, tumor nests consist of dark, blue cells at the periphery, which often have a palisade arrangement. Somewhat larger cells of lighter cytoplasm occupy the central parts. Myoepithelial differentiation exists in all variants and it is not obvious on standard H&E preparations, however, the immunohistochemical analysis has proven it [24]. The differential diagnosis of basal cell adenoma includes polymorphous low-grade adenocarcinoma and pleomorphic adenoma, as well as some of myoepithelioma variants. The immunohistochemical analysis of our results, in accordance with data from the literature, showed that cells at the periphery of tumor nests express p63, vimentin, SMA and S100, whereas cells in the interior or luminal cells, if we talk about the tubular variant, were positive to SK14, CEA and S100.

Analizirajući ekspresiju markera, dolazimo do zaključka da je adenom bazalnih ćelija histogenetski poreklom od ćelija interkalatnih kanala [25].

Evaluacija mitotskog indeksa, pre svega Ki67 proliferativnog indeksa, pokazala se kao veoma bitan parametar u diferencijalnoj dijagnostici, predviđanju biološkog ponašanja i agresivnosti u mnogim tumorima. Našim istraživanjem smo obuhvatili 32 tumora pljuvačnih žlezda, benignih i malignih, analizirali smo proliferacioni indeks i u korelaciji sa ostalim kliničko-patološkim karakteristikama dobili smo značajne rezultate u diferencijalnoj dijagnostici ovih neoplazmi.

Za potrebe našeg istraživanja koristili smo 5% kao graničnu vrednost za Ki67 proliferativni indeks, ali u dostupnoj literaturi postoje studije koje su se vodile višim vrednostima za agresivnost tumora, i to >10% [26,27]. U našem istraživanju je najveću vrednost pokazao celularni rekurentni pleomorfni adenenom mekog nepca.

Slično rezultatima našeg istraživanja, Shida i saradnici su dobili nešto veće vrednosti Ki67 proliferativnog indeksa u grupi adenoma bazalnih ćelija [28]. Sa rezultatima Horii i saradnika poklapaju se naši rezultati, koji ukazuju na najveću proliferativnu aktivnost pleomorfnog adenoma u grupi benignih tumora [29]. Upoređujući ekspresiju p53 i Ki67, Saghravanian i saradnici navode da je od velikog značaja u diferencijaciji polimorfnog adenokarcinoma niskog gradusa i adenoidnocističnog karcinoma. U njihovoj studiji je 24% jedara tumorskih ćelija pokazalo ekspresiju Ki67, dok je svega 3,88% u grupi polimorfnog adenokarcinoma niskog gradusa. Na osnovu velike varijabilnosti dobijenih rezultata u našem istraživanju, a i od strane drugih istraživača, po našem mišljenju, jedan je od dokaza da niska vrednost proliferativnog indeksa nije uvek strogo povezana sa tumorom niskog gradusa. rezultati našeg istraživanja pokazuju da vrednost Ki67 proliferativnog indeksa varira u zavisnosti od slučaja do slučaja, kao i od histološkog tipa samog tumora.

Ispitivanje ekspresije p53 i Ki67 je široko zastupljena metoda u utvrđivanju evolucije i prognoze malignih tumora svih lokalizacija. Najčešće identifikovani mutirani gen u malignim neoplazmama je p53, naročito je zastupljen kod karcinoma dojke, želuca, jetre i prostate. Ekspresija p53 je udružena sa negativnom prognozom bolesti, i obično je prisutna kod agresivnih malignih tumora visokog gradusa. Mutacija gena je uočena i kod tumora pljuvačnih žlezda, ali su rezultati još uvek nedovoljni i nepotpuni.

Having analyzed the expression of markers, we concluded that basal cell adenoma was histogenetically of intercalated channel cells origin [25].

The evaluation of the mitotic index, primarily the Ki67 proliferative index, proved to be a very important parameter in the differential diagnosis, predicting biological behavior and aggressiveness in many tumors. Our study included 42 salivary gland tumors, both benign and malignant. We analyzed the proliferative index and, in correlation with other clinical-pathological characteristics, we obtained significant results in the differential diagnosis of these neoplasms.

For the purposes of our study, we used 5% as a threshold for the Ki67 proliferative index, but the existing literature offers studies which were conducted using higher values for tumor aggressiveness, i.e. >10% [26,27]. In our study, recurrent cellular pleomorphic adenoma of the soft palate showed the highest value.

Similar to the results of our study, Shida et al. obtained slightly higher values of the Ki67 proliferative index in the basal cell adenoma group [28]. Our results match the results of Horii et al., which indicate the highest proliferative activity of pleomorphic adenoma in the group of benign tumors [29]. By comparing the expressions of p53 and Ki67, Saghravanian et at. state that they are of great importance for the differentiation of polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma. In their study, 24% of tumor cell nuclei showed the expression of Ki67, contrary to only 3.88% in the polymorphous low-grade adenocarcinoma group. Based on great variability of the obtained results in our study, as well as results from other researchers, we believe that a low value of the proliferative index is not necessarily always strictly related to a low-grade tumor. The results of our study show that the value of the Ki67 proliferative index varies depending on the case and the histological type of the tumor itself.

The examination of the expression of p53 and Ki67 is a widespread method in the determination of the evolution and prognosis of malignant tumors of all localizations. p63 is the most commonly identified mutated gene in malignant neoplasms, especially present in breast, stomach, liver and prostate cancer. The expression of p53 is associated with the negative prognosis of a disease, and it is usually present in aggressive malignant

Weber je sa svojim saradnicima analizirao ekspresiju p53 u grupi benignih tumora. Pokazali su da ekspresija proteina postoji jedino u grupi pleomorfnog adenoma i mioepitelioma. Svoje rezultate su tumačili kao povećanu sklonost tumora ka malignoj alteraciji [30].

Korišćenjem softverskog paketa „ImageJ" analizirano je šest jedarnih parametara: površina, perimetar, cirkularnost, zaobljenost, Feretov dijametar i integrisana optička gustina.

Razlike nađene za jedarne parametre, koji se odnose na veličinu jedara (površina i perimetar), kao i na oblik jedra (cirkularnost i zaobljenost), minimalno su bile veće u grupi polimorfnog adenokarcinoma niskog gradusa. Vrednosti za Feretov dijametar i integrisane optičke gustine statistički značajno zavise od prirode i tipa tumora ($p<0,05$).

U nama dostupnoj literaturi, generalno, jako je malo studija koje su se bavile morfometrijskim karakteristikama tumora. Već svetlosnom mikroskopijom možemo da uočimo neke od karakteristika jedara. Jasno se vidi da li je reč o hiperhromnim jedrima, krupnim ili malim, da li je prisutna atipija, kakav je hromatin, da li su ivice ravne, površina nazupčana. Diferencijalna dijagnoza tumora pljuvačnih žlezda je često veoma teška, a morfometrija omogućava kvantifikaciju patohistološkog nalaza, a samim tim i smanjuje mogućnost greške u postavljanju definitivne dijagnoze. Morfometrijska analiza tumora pljuvačnih žlezda je do sada primenjivana i mali je broj studija koje su komparirale rezultate ovih analiza u različitim tipovima tumora. U dosadašnjim istraživanjima autori su uglavnom istraživali jedarnu površinu, cirkularnost, perimetar i integrisanu optičku gustinu, korišćenjem različitih softverskih paketa [31,32]. Layfield je u svojoj studiji objavio rezultate vezane za ove parametre i pokazao njihovu dijagnostičku značajnost u diferencijaciji benignih i malignih mešovitih tumora parotidne žlezde [33]. Prvulović je sa svojim saradnicima objavila studiju u diferencijaciji duktalnog i lobularnog karcinoma dojke na citološkom materijalu. Takođe je istakla i primenjivost ove metode, ne samo u diferencijaciji, već i u gradiranju samog karcinoma [34]. Oz i saradnici su objavili studiju koja se bavila morfometrijskim karakteristikama ćelija oralne sluzokože kod bolesnika sa dijabetesom

high-grade tumors. Gene mutation has also been noticed in salivary gland tumors, but the results are still insufficient and incomplete.

Weber et al. analyzed the expression of p53 in the benign tumor group.
They showed that the expression of protein existed only in the pleomorphic adenoma and myoepithelioma groups. They interpreted their results as an increased tendency of tumors to malignant alteration [30].

Six nuclear parameters were analyzed using the software pack "ImageJ": area, perimeter, circularity, roundness, Feret diameter and integrated optical density.

The differences found for the nuclear parameters which refer to the size of nuclei (area and perimeter), as well as the shape of nuclei (circularity and roundness), were slightly higher in the polymorphous low-grade adenocarcinoma group. The values of Feret diameter and integrated optical density significantly depend on the nature and type of the tumor ($p < 0.05$).

The available literature offers a very small number of studies dealing with the morphometric characteristics of tumors. Using light microscopy, we can notice some of the characteristics of nuclei. It can be clearly seen whether it is the case of hyperchromatic nuclei, large or small, whether atrophy is present, what chromatin is like, whether the edges are even, or the area serrated. The differential diagnosis of salivary gland tumors is often quite difficult, and the morphometry enables the quantification of a pathohistological finding, and therefore reduces the possibility for errors in setting the final diagnosis. The morphometric analysis of salivary gland tumors has been applied so far, but the number of studies which have compared the results of these analyses in different tumor types is rather small. In previous studies, the authors mostly examined nuclear area, circularity, perimeter and integrated optical density using different software packs [31,32]. Layfield published the results for these parameters in his study, and showed their diagnostic significance in the differentiation of benign and malignant mixed tumors of the parotid gland [33]. Prvulović et al. published a study on the differentiation of ductal and lobular breast carcinoma on cytological material. They also emphasized the applicability of this method, not only in the differentiation, but also in grading the ca-

melitusom tip I, i pokazali kako su jedarni parametri statistički veći u ovoj grupi bolesnika [35]. Obad-Kovačević je u svoje istraživanje uključila morfometrijske karakteristike benignih i malignih tumora parotidne žlezde, proučavajući karakteristike cele ćelije. Došli su do zaključka da je odnos površine jedara i citoplazme znatno veći, u korist jedara, kod malignih tumora [36].

Zaključak

Plemorfni adenomi su pozitivni na S-100, GFAP, CK14, αSMA, CEA, EMA i WT1. Adenom bazalnih ćelija pokazuje pozitivnost na S-100, CEA, p63 i vimentin. Ekspresija HER2 u grupi benignih tumora bila je u potpunosti odsutna. Polimorfni adenokarcinom niskog gradusa pokazao je pozitivnu imunohistohemijsku reakciju na CK14, p63, EMA, S100 i vimentin.

Najveću proliferativnu aktivnost pokazuje pleomorfni adenom. Dobijeni rezultati su u skladu sa frekvencijom recidi-viranja tumora, kao i sa stepenom maligne alteracije.

Vrednosti morfometrijskih parametara, integrisana optička gustina i Feretov dijametar statistički su veće u grupi polimorfnog adenokarcinoma niskog gradusa. U grupi adenoma bazalnih ćelija vrednosti za integrisanu optičku gustinu statistički su veće u odnosu na grupu pleomorfnih adenoma.

rcinoma itself [34]. Oz et al. published a study on the morphometric characteristics of oral mucosa cells in patients with diabetes mellitus type I, and showed that nuclear parameters were higher in this group of patients [35]. Obad-Kovačević et al. included morphometric characteristics of benign and malignant tumors of the parotid gland in their research by studying the characteristics of the entire cell. They concluded that the ratio of the area of nuclei and cytoplasm was considerably higher in malignant tumors, in favor of nuclei[36].

Conclusion

Pleomorphic adenoma was positive to S-100, GFAP, CK14, αSMA, CEA, EMA and WT1, whereas basal cell adenomas showed positivity to S-100, CEA, p63 and vimentin. The expression of HER2 in the benign tumor group was completely absent. Polymorphous low-grade adenocarcinoma showed a positive immunohistochemical reaction to CK14, p63, EMA, S100 and vimentin.

Pleomorphic adenoma showed the highest proliferative activity. The obtained results were in accordance with the frequency of tumor recurrence, as well as the degree of malignant alteration.

The values of morphometric parameters – integrated optical density and Feret diameter – were statistically higher in the polymorphous low-grade adenocarcinoma group. The basal cell adenoma group showed statistically higher values for integrated optical density compared to the pleomorphic adenoma group.

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